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Functional characterization and Me^{2+} ion specificity of a Ca^{2+}–citrate transporter from *Enterococcus faecalis*

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Keywords
CITMHS family; citrate fermentation; citrate transport; *Enterococcus faecalis*; Me–citrate complex

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Secondary transporters of the bacterial CitMHS family transport citrate in complex with a metal ion. Different members of the family are specific for the metal ion in the complex and have been shown to transport Mg^{2+}–citrate, Ca^{2+}–citrate or Fe^{3+}–citrate. The Fe^{3+}–citrate transporter of *Streptococcus mutans* clusters on the phylogenetic tree on a separate branch with a group of transporters found in the phylum Firmicutes which are believed to be involved in anaerobic citrate degradation. We have cloned and characterized the transporter from *Enterococcus faecalis Ef*CitH in this cluster. The gene was functionally expressed in *Escherichia coli* and studied using right-side-out membrane vesicles. The transporter catalyzes proton-motive-force-driven uptake of the Ca^{2+}–citrate complex with an affinity constant of 3.5 μM. Homologous exchange is catalyzed with a higher efficiency than efflux down a concentration gradient. Analysis of the metal ion specificity of *Ef*CitH activity in right-side-out membrane vesicles revealed a specificity that was highly similar to that of the *Bacillus subtilis* Ca^{2+}–citrate transporter in the same family. In spite of the high sequence identity with the *S. mutans* Fe^{3+}–citrate transporter, no transport activity with Fe^{3+} (or Fe^{2+}) could be detected. The transporter of *E. faecalis* catalyzes translocation of citrate in complex with Ca^{2+}, Sr^{2+}, Mn^{2+}, Cd^{2+} and Pb^{2+} and not with Mg^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+}. The specificity appears to correlate with the size of the metal ion in the complex.

Analysis of a large set of bacterial genomes has shown that, in spite of its high abundance in nature, only a limited number of bacteria are able to ferment citrate under anoxic conditions [1]. All known fermentative pathways for citrate use citrate lyase as the first metabolic enzyme, and the genes coding for the lyase are easily recognized on the genomes. Of 156 genomes analyzed, only 19 contained the citrate lyase genes, most of them either from the γ-subdivision of the Proteobacteria or the Bacillales and Clostridia of the Firmicutes. Despite the low spread, there was a remarkable diversity in the pathways in terms of sensory systems for detection of the substrates, enzymes used for metabolic steps, energy conservation in the pathways, and the transporters responsible for the uptake of citrate from the medium. Transporters from four different gene families were identified in the gene clusters. The Proteobacteria use Na^+-gradient-driven citrate transporters from the 2-hydroxycarboxylate transporter (2HCT) family (TC 2.A.24. CCS [2,3]), whereas Gram-positive bacteria use citrate/lactate exchangers from the same family. Transporters from the DASS family (TC 2.A.47), which are believed to be citrate/succinate antiporters [4], are also involved in

Abbreviations
CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PMF, proton motive force; RSO, right-side-out.

both phyla. In addition, the citrate fermentation cluster of *Clostridium tetani* contains a gene coding for a transporter from an uncharacterized family (TC 9.B.50), and the clusters of the three lactic acid bacteria *Streptococcus mutans*, *Streptococcus pyogenes* and *Enterococcus faecalis* contain genes coding for transporters of the CitMHS family (TC 2.A.11). Remarkably, the four families are found in the same structural class (ST [3]) in the MemGen classification system of membrane proteins, suggesting a common fold and evolutionary origin [1,5].

In contrast with most citrate transporters, characterized members of the CitMHS family transport citrate in complex with a bivalent metal ion. This makes sense when citrate in the environment of the organism would mostly be available in the metal-ion-complexed state. The best-characterized members of the family are two transporters from the soil bacterium *Bacillus subtilis*, *Bs*CitM and *Bs*CitH. The former transports citrate in complex with Mg^{2+} and is the major citrate-uptake system during growth on citrate under aerobic conditions [6–9]. *Bs*CitH shares 61% sequence identity with *Bs*CitM, but transports the complex of citrate with Ca^{2+} [7]. The physiological function of *Bs*CitH is unknown. The CitMHS family of transporters contains over 60 members, all of bacterial origin. The phylogenetic tree of the family reveals that the three members associated with the fermentative citrate pathways of *S. mutans*, *S. pyogenes* and *E. faecalis* are on a separate branch of the tree that is well separated from other branches (Fig. 1). The transporters of *Lactobacillus* species *casei* and *sakei*, which are on the same branch, are also associated with the citrate lyase genes on the genomes, suggesting that the branch is specific for citrate fermentation pathways in lactic acid bacteria. The transporters on the branch share 75–83% sequence identity. Recently, it was reported that *Sm*CitM of *S. mutans* catalyzes the uptake of citrate in complex with Fe^{3+} [10]. The result suggests that the physiological function of the transporters may not always be the uptake of citrate that is simply available in the Mg^{2+} or Ca^{2+} complexed state in the environment, but also the uptake of the complexed metal ion. The authors suggested the relevance of Fe^{3+}–citrate uptake in iron homeostasis which may play a significant role in the pathogenesis of *S. mutans*.

Here we report on the catalytic properties of *Ef*CitH, the transporter coded in the citrate fermentation cluster of *E. faecalis*. Surprisingly, and in spite of the high sequence identity with the *Sm*CitM of *S. mutans*, it is demonstrated that *Ef*CitH transports Ca^{2+}–citrate and has a metal ion specificity that is very similar to that observed for *Bs*CitH of *B. subtilis*.

Results

Functional characterization of CitH of *E. faecalis*

Citrate transport by the gene product of *EfcitH* located in the citrate fermentation operon of *E. faecalis* ATCC29212 was demonstrated by comparing the uptake of [1,5-^{14}C]citrate in right-side-out (RSO) membrane vesicles prepared from cells of *Escherichia coli* BL21 containing either pET-*Ef*citH or the control vector pET28b, both induced with 0.25 mM isopropyl β-D-thiogalactopyranoside. The membranes were energized using the artificial electron donor system ascorbate/phenazine methosulfate (see Experimental procedures). At a concentration of 4.4 μM [1,5-^{14}C]citrate, the vesicles prepared from the control cells were essentially devoid of uptake activity in line with the lack of an endogenous *E. coli* citrate transporter (Fig. 2A, □). RSO membrane vesicles containing *Ef*CitH took up citrate at a low but significant rate [0.25 pmol·s^{-1}·(mg membrane protein)$^{-1}$], demonstrating functional expression of the cloned gene (●). No uptake was observed in the absence of the energizing system (not shown). The initial rate of uptake was reduced to the level observed with the control membranes in the presence of 1 mM EDTA (▼), and addition of Ca^{2+} in excess of EDTA resulted in an increase in the initial rate of uptake by one order of magnitude (compare ■ and ●). The results suggest that the complex of Ca^{2+} and citrate is the true substrate of *Ef*CitH and that the low uptake in the absence of added Ca^{2+} was due to contaminating free Ca^{2+} in the assay buffers which could effectively be removed by EDTA. To exclude adverse effects of Ca^{2+} or EDTA on the (energetic) state of the membranes, the uptake of L-[4-^{14}C]proline by the same membranes containing *Ef*CitH was studied under identical conditions. The uptake of L-[4-^{14}C]proline was not affected in the presence of 1 mM EDTA, while the excess of 2 mM Ca^{2+} had a slight stimulatory effect on the initial rate of uptake (Fig. 2B).

The kinetic parameters for Ca^{2+}–citrate uptake catalyzed by *Ef*CitH were estimated from a series of uptake experiments in which the total Ca^{2+} concentration was fixed at 1.5 mM and the [1,5-^{14}C]citrate concentration was varied between 0.55 and 8.8 μM. The corresponding range of Ca^{2+}–citrate concentrations was 0.5–7.5 μM. The initial rates of uptake by the RSO membrane vesicles revealed that the transporter had a high affinity for the complex with a K_m of 3.5 μM. The maximal rate was estimated to be 2.05 nmol·min^{-1}·(mg membrane protein)$^{-1}$ (not shown).

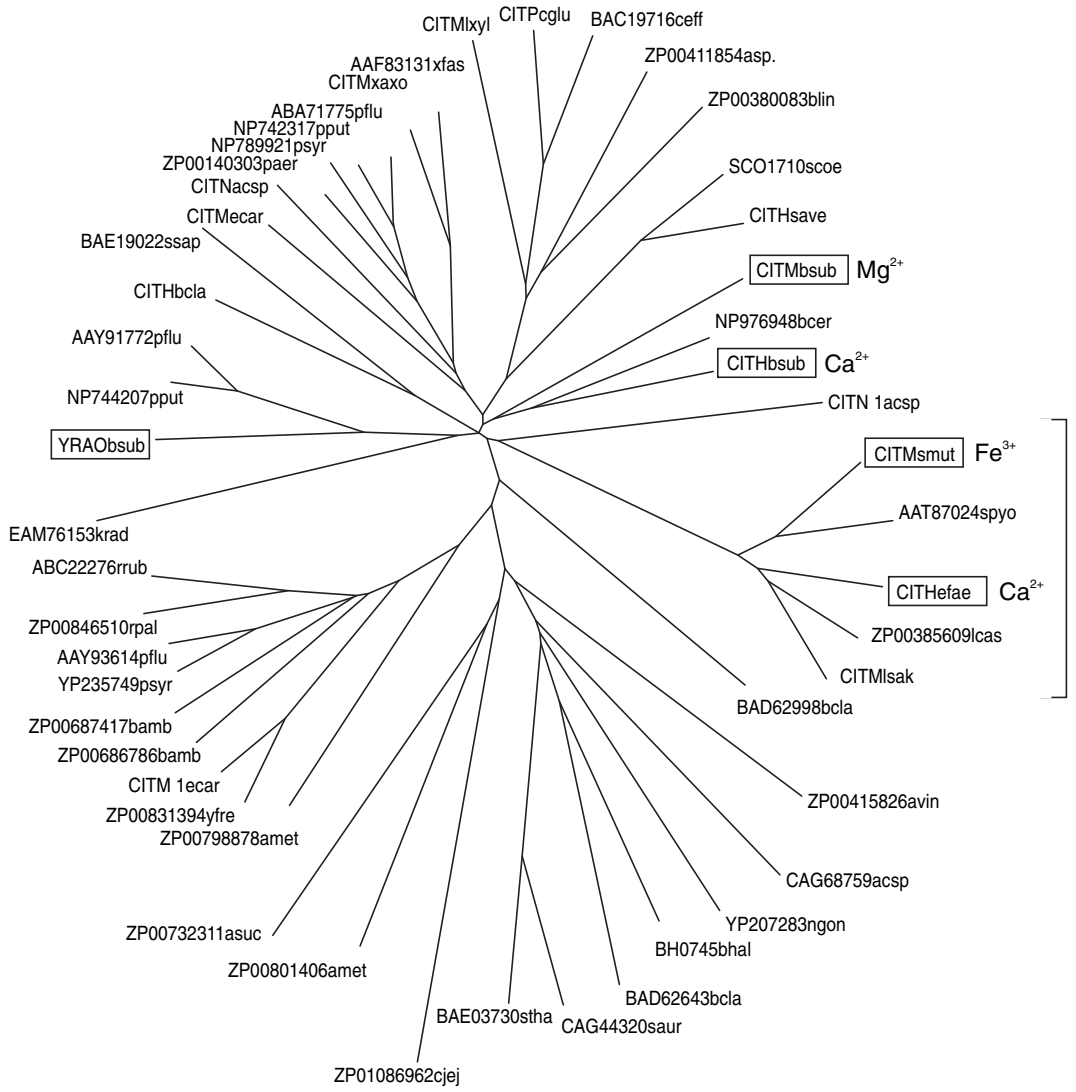

Fig. 1. Phylogenetic tree of the CitMHS family. Unrooted tree of 92 members of the CitMHS family in structural class ST [3] in the MemGen classification (family [st301]MeCit). Details on the individual members can be found at our website (http://molmic35.biol.rug.nl/memgen/mgweb.dll). Sequences with sequence identities higher than 90% were removed from the tree. A multiple sequence alignment was computed using CLUSTAL W [24]. The five transporters discussed in this paper, EfCitH (CITHefae), SmCitM (CITMsmut), BsCitH (CITHbsub), BsCitM (CITMbsub) and YRAObsub, are boxed, and the bi/trivalent metal ion specificity is indicated. The specificity of the CITHefae transporter is based on the present study.

Homologous exchange catalyzed by *Ef*CitH was demonstrated by chase experiments (Fig. 3). Membrane vesicles containing *Ef*CitH were allowed to accumulate [1,5-^{14}C]citrate for 5 min, driven by the proton gradient and in the presence of Ca^{2+}. Addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which kills the proton gradient instantaneously, resulted in slow efflux of label from the membranes down the concentration gradient (▼). The presence of excess external EDTA did not effect the efflux process, as expected (◆). Addition of 500 μM citrate together with CCCP resulted in a much faster release of label, indicative of homologous exchange catalyzed by *Ef*CitH (■).

The results demonstrate the functional expression of the *EfcitH* gene in *E. coli* and identify the gene product as a proton-motive force (PMF)-driven, high-affinity transporter for the Ca^{2+}–citrate complex.

Heterologous expression of CitH of *E. faecalis*

Heterologous expression of the *citH* gene of *E. faecalis* proved to be very difficult. A number of different vectors containing the gene with N-terminal or C-terminal

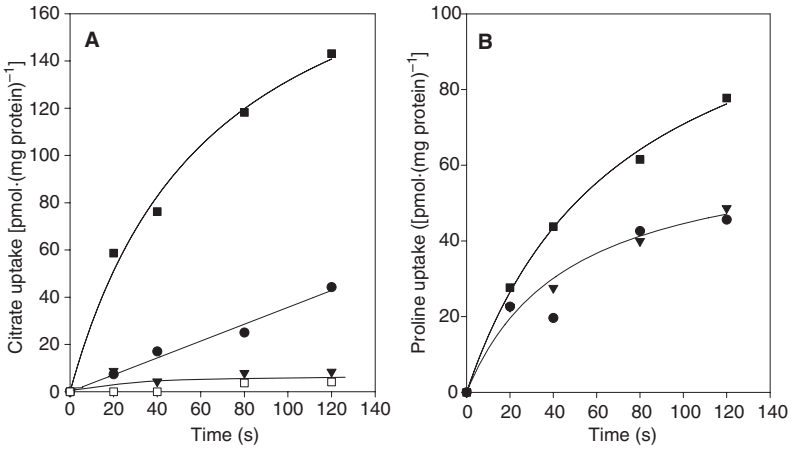

Fig. 2. Citrate and proline uptake by RSO membrane vesicles. RSO membrane vesicles were prepared from *E. coli* BL21(DE3) harboring plasmid pET28b (□) or pET-*Ef*citH (closed symbols). (A) [1,5-^{14}C]citrate uptake in the absence (●,□) or presence of 1 mM EDTA (▼), and 1 mM EDTA + 2 mM Ca^{2+} (■). (B) L-[4-^{14}C]proline uptake in the absence (●) or presence of 1 mM EDTA (▼), and 1 mM EDTA + 2 mM Ca^{2+} (■).

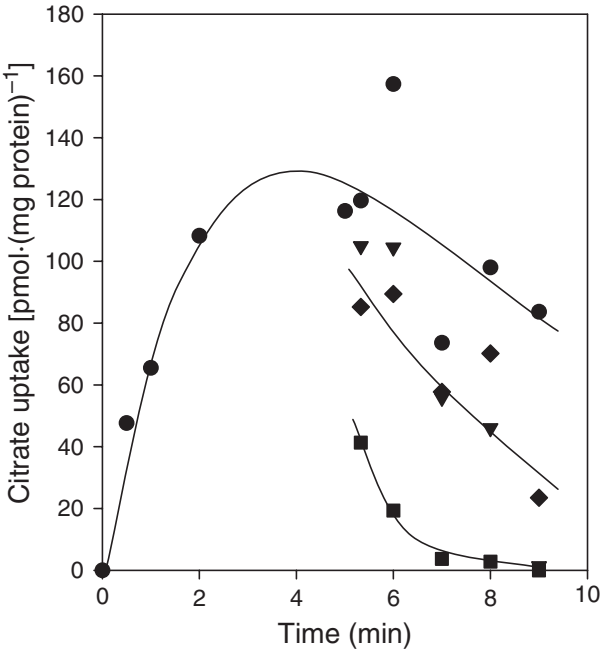

Fig. 3. Chase experiments in *Ef*CitH RSO membrane vesicles. RSO membranes prepared from *E. coli* BL21(DE3) harboring plasmid pET-*Ef*citH were allowed to take up [1,5-^{14}C]citrate for 5 min, after which buffer (●), 10 μM CCCP (▼), 10 μM CCCP + 1 mM EDTA (◆) or 10 μM CCCP + 0.5 mM citrate (■) was added.

extensions coding for an enterokinase site and 6 consecutive histidine residues (His-tag) or just a His-tag were constructed and transformed to different *E. coli* strains. Also, the gene was cloned in the nisin-inducible NICE system for expression in the related Gram-positive bacterium *Lactococcus lactis* [11,12]. The different combinations of vectors and strains were tested under various growth conditions, but only the above combination of the pET-*Ef*citH vector in *E. coli* BL21(DE3) resulted in detectable expression. In all cases, including the latter, immediate growth arrest was observed after induction. Moreover, no produced protein could be detected by immunoblotting using antibodies directed against the His-tag for any of the combinations, which may be due to low expression levels or to processing of the His-tag. The lack of detection of both the constructs with the N-terminal and C-terminal His-tag suggested the former. As an alternative, successful expression was detected by [1,5-^{14}C]citrate uptake by whole cells.

The immediate growth arrest upon expressing the *Ef*CitH protein suggested that the protein is extremely harmful to the host cell. Comparison of the uptake of L-[4-^{14}C]proline in RSO membrane vesicles prepared from *E. coli* BL21(DE3) harboring the pET28b and pET-*Ef*citH plasmids strongly suggested that the protein negatively affects the integrity of the membranes or the energetic state of the vesicles. Membranes containing the *Ef*CitH protein revealed a 10 times lower proline uptake activity than the control membranes (Fig. 4). As a consequence, the uptake rate catalyzed by the *Ef*CitH protein as observed in Fig. 2A is, in comparison with uptake rates by other secondary transporters, likely to be greatly underestimated because the expression level is below the detection limit and the energetic state of the membrane is very poor.

Metal ion specificity of CitH of *E. faecalis*

The metal ion specificity in the Me–citrate complex transported by *Ef*CitH was determined using the protocol for Ca^{2+}–citrate uptake demonstrated in Fig. 2A. Contaminating metal ions in the buffer were complexed to EDTA, after which an excess of various bivalent metal ions over EDTA was added to drive citrate in the desired complex. In view of the poor condition of the membranes expressing *Ef*CitH (Fig. 4) and

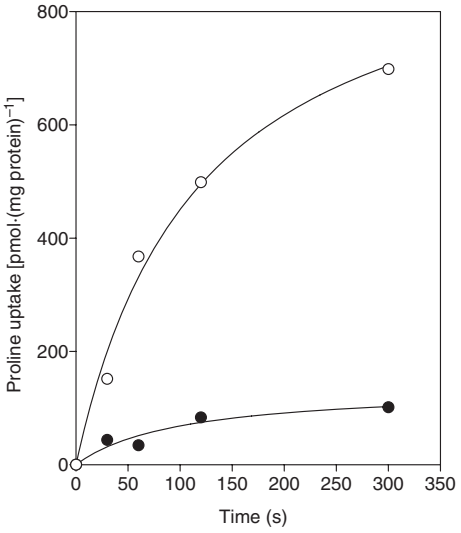

Fig. 4. Effect of *Ef*citH expression on proline uptake by RSO membranes. L-[4-^{14}C]Proline uptake was measured in RSO membrane vesicles prepared from *E. coli* BL21(DE3) harboring plasmid pET28b (○) or pET-*Ef*citH (●).

the toxicity of many of the ions tested, the effect of the latter was first analyzed on L-[4-^{14}C]proline uptake both by the membranes containing *Ef*CitH and the control membranes to exclude effects not related to the transporter (Fig. 5).

On the whole, the effects of the various metal ions on proline uptake by the two types of membrane were comparable, indicating that, in spite of their poor condition, the membranes containing *Ef*CitH were not more sensitive to the presence of the metal ions than the endogenous membranes. In fact, the control membranes appeared to be slightly more sensitive. Different ions clearly exerted different effects. Mg^{2+}, Mn^{2+} and Pb^{2+} had a stimulatory effect on the uptake rate, in particular in the case of the *Ef*CitH membranes, Ca^{2+}, Ba^{2+}, Sr^{2+} and Co^{2+} showed only marginal effects, Zn^{2+}, Ni^{2+} and Cd^{2+} inhibited the uptake by 50–70%, and Cu^{2+} completely inhibited the uptake of proline. Cd^{2+} appeared to be more inhibitory in the *Ef*CitH membranes than in the control membranes.

Uptake of citrate by the control membranes showed that the presence of some of the metal ions, especially Cd^{2+} and Pb^{2+}, increased the background of the transport assay (Fig. 6). Significantly higher uptakes of citrate by the membranes containing *Ef*CitH were observed in the presence of Ca^{2+}, Sr^{2+}, Cd^{2+} and Pb^{2+}. A low activity above background was observed with Mn^{2+}, while no uptake was observed with Ba^{2+}, Zn^{2+}, Ni^{2+}, Mg^{2+}, Co^{2+} and Cu^{2+} (Fig. 6). In spite of the partial inhibition of proline transport observed for Zn^{2+} and Ni^{2+}, the conclusion that these ions are not transported by *Ef*CitH appears to be confirmed. For Cu^{2+}, the result is clearly inconclusive in view of the complete inhibition of proline uptake by Cu^{2+}.

The homologous protein from *S. mutans* (75% sequence identity) has been reported to transport citrate in complex with Fe^{3+} [10]. Significant uptake of

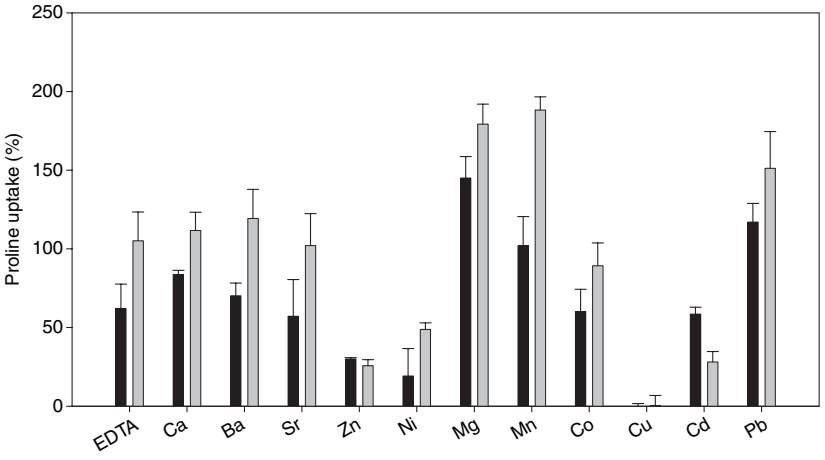

Fig. 5. Effect of bivalent metal ions on proline uptake by RSO membrane vesicles. L-[4-^{14}C]Proline uptake by RSO membrane vesicles prepared from *E. coli* BL21(DE3) harboring plasmid pET28b (solid bars) or pET-*Ef*citH (grayed bars) was measured after 1 min incubation with 1.7 μM L-[4-^{14}C]proline in the presence of 1 mM EDTA and an excess of the indicated bivalent cation. Ca^{2+}, Ba^{2+}, Sr^{2+}, Zn^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+} were added at a final concentration of 2 mM. Cu^{2+}, Cd^{2+} and Pb^{2+} were added to a final concentration of 1.1 mM. Uptake was expressed as a percentage of the uptake obtained in a buffer without EDTA and bivalent metal ions, which corresponded to 139.3 ± 20.6 and 15.9 ± 1.8 pmol·(mg protein)$^{-1}$ for the control and *Ef*CitH-expressing membranes, respectively. Error bars represent the standard deviation of triplicate measurements.

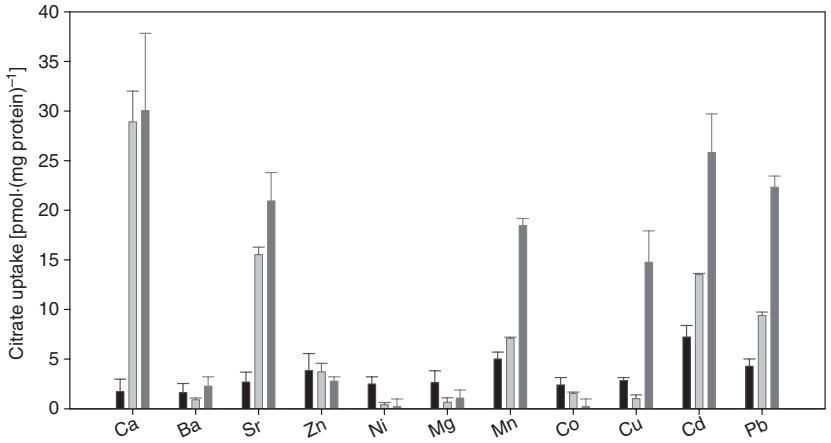

Fig. 6. Metal ion specificity of *Ef*CitH and *Bs*CitH in RSO membranes. [1,5-^{14}C]Citrate uptake by RSO membrane vesicles prepared from *E. coli* BL21(DE3) harboring plasmid pET28b (solid bars), pET-*Ef*citH (light gray bars), or pWSKcitH (dark gray bars) was measured after 1 min incubation with 4.4 μM [1,5-^{14}C]citrate in the presence of 1 mM EDTA and an excess of the indicated bivalent cations. The cations Ca^{2+}, Ba^{2+}, Sr^{2+}, Zn^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+} and Co^{2+} were added at a final concentration of 2 mM, and Cu^{2+}, Cd^{2+} and Pb^{2+} were added at a final concentration of 1.1 mM. Error bars represent the standard deviation of triplicate experiments.

Table 1. Citrate and proline uptake activity of RSO membrane vesicles in the presence of Fe^{2+} and Fe^{3+}. Experiments were performed as described in the legends of Figs 3 and 4. The buffer contained 4.4 μM [1, 5-^{14}C]citrate and 75 μM Fe^{2+} or Fe^{3+} final concentrations. The rate of proline uptake is expressed as the percentage of the rate in the absence of the metal ions. ND, not determined.

	L-[4-^{14}C]Proline uptake (%)		[1,5-^{14}C]Citrate retained [pmol·(mg protein)$^{-1}$]	
	Fe^{3+}	Fe^{2+}	Fe^{3+}	Fe^{2+}
Control membranes	57.1 ± 3.4	92.6 ± 13.1	9.1 ± 4.0	7.0 ± 2.6
*Ef*CitH membranes	73.5 ± 16.5	84.2 ± 1.57	12.2 ± 2.7	9.2 ± 2.3
*Bs*CitH membranes	ND	ND	9.1 ± 0.5	6.9 ± 4.3

[1,5-^{14}C]citrate was observed by whole cells of *S. mutans* at a concentration of 4.4 μM citrate and 1 μM Fe^{3+}. Using exactly the same conditions, the membranes containing *Ef*CitH did not take up [1,5-^{14}C]citrate (not shown). Under these experimental conditions, the concentration of the Fe^{3+}–citrate complex was only 0.3 μM. Increasing the Fe^{3+} concentration to 75 μM gives a Fe^{3+}–[1,5-^{14}C]citrate concentration of 3.9 μM. Proline uptake experiments revealed a small negative effect on the rate under these conditions, while the increase in the background of the citrate uptake assay was still acceptable (Table 1). No uptake of [1,5-^{14}C]citrate by membranes containing *Ef*CitH was observed under these conditions (Table 1), and the same results were obtained with bivalent Fe^{2+}. It is concluded that neither Fe^{2+}–citrate nor Fe^{3+}–citrate are substrates of *Ef*CitH in RSO membrane vesicles.

The metal ion specificity of *Ef*CitH resembles the specificity of the homologous transporter *Bs*CitH of *B. subtilis* which was reported to transport citrate in complex with Ca^{2+}, Sr^{2+} and Ba^{2+} based on studies using whole cells [7]. The specificity of *Bs*CitH was re-examined in RSO membranes using the experimental conditions reported here for *Ef*CitH. The effect of the various metal ions on proline transport in membranes expressing *Bs*CitH was similar to that described above for the other membranes (not shown). Both transporters mediated the uptake of citrate in complex with Ca^{2+}, Sr^{2+} Cd^{2+} and Pb^{2+} and not with Ba^{2+}, Zn^{2+}, Ni^{2+}, Mg^{2+}, and Co^{2+} (Fig. 6). Also, the *Bacillus* transporter did not seem to have affinity for the Fe^{2+}–citrate or Fe^{3+}–citrate complex (Table 1).

Discussion

The genetic organization of the citrate fermentation clusters on the genomes of *E. faecalis* and *S. mutans* are similar, but not the same. Upstream of the *citDEF* genes coding for the α, β and γ subunits of citrate lyase are the *oadDB* genes coding for the δ and β subunits

of the membrane-bound oxaloacetate decarboxylase and the divergently transcribed genes coding for the putative citrate transporter. The citrate lyase accessory gene *citX* and the *oadA* gene coding for the α subunit of the decarboxylase are located downstream of the citrate lyase genes. The clusters differ in the location of two additional citrate lyase accessory genes, *citC* and *citG*, and, most remarkably, in the presence of a second oxaloacetate decarboxylase gene, also named *citM*, that is only found in the *E. faecalis* cluster. The latter gene codes for a different type of oxaloacetate decarboxylase that belongs to the malic enzyme family [13]. The differences suggest that the physiology of the gene cluster may not be exactly the same in both organisms. Nevertheless, it was a surprise to find that the substrate specificity of the closely related transporters in the two clusters was not the same. It was demonstrated that the citrate uptake activity of *Ef*CitH of *E. faecalis* was strictly dependent on the presence of bivalent metal ions, as the addition of EDTA completely abolished uptake. The presence of Ca^{2+} resulted in the highest uptake activity, suggesting that under physiological conditions *Ef*CitH functions as a Ca^{2+}–citrate transporter. *Sm*CitM of *S. mutans* has been reported to transport Fe^{3+}–citrate [10], a complex that clearly was not a substrate of *Ef*CitH.

The metal ion specificity of the *Ef*CitH transporter mostly resembles that of the *Bs*CitH transporter of *B. subtilis* with which it shares 44% sequence identity. Uptake studies in RSO membranes containing the transporters revealed transport of citrate in complex with Ca^{2+}, Sr^{2+}, Mn^{2+}, Cd^{2+} and Pb^{2+} and not with Mg^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+}. *Bs*CitH showed in addition activity with Cu^{2+}–citrate (see below). Complexes of citrate with the group of metal ions that are not transported by *Ef*CitH and *Bs*CitH are substrates of a second transporter of the CitMHS family found in *B. subtilis*, *Bs*CitM [7]. The ability to take up toxic bivalent metal ions in complex with citrate is a serious threat for an organism. The presence of Zn^{2+} and Co^{2+} in citrate-containing medium was shown to be extremely toxic to *B. subtilis* under conditions in which *Bs*CitM was expressed [14]. This may be the reason for the strict regulation of expression of the transporter, which involves a number of regulatory systems. Expression is repressed by carbon catabolite repression [15] and by arginine metabolism [16], and induced by a two-component sensory system [15,17]. Moreover, the expression of the latter is itself under control of carbon catabolite repression [18]. *B. subtilis* and *E. faecalis* will be at a similar risk in citrate-containing medium in the presence of Cd^{2+} or Pb^{2+} when *Ef*CitH and *Bs*CitH are expressed.

*Ef*CitH of *E. faecalis* and *Sm*CitM of *S. mutans* are very similar proteins sharing 75% sequence identity. Uptake studies in RSO membranes presented here show that *Ef*CitH is a Ca^{2+}–citrate transporter, while uptake studies in whole cells have demonstrated that *Sm*CitM is a Fe^{3+}–citrate transporter [10]. To exclude artefacts caused by the different experimental systems, the specificity of *Ef*CitH was confirmed in whole cells (not shown). Unfortunately, attempts to express the *S. mutans* transporter in *E. coli* or *L. lactis* failed. Consequently, the specificity of *Sm*CitM could not be determined in RSO membranes. Heterologous expression of genes from the CitMHS family appears to be problematic in general, as previous attempts to express a third gene of *B. subtilis*, *yraO*, from the same family failed (unpublished results), and *Bs*CitH, *Bs*CitM, and *Ef*CitH are only produced at low levels when very specific vector/host combinations are used. Expression of the genes appears to be extremely toxic, as the cells cease to grow immediately upon induction. The dramatic decrease in proline uptake activity in RSO membranes containing *Ef*CitH (Fig. 4) suggests that insertion of a low quantity of protein already dramatically affects the state of the membrane. To date there is no explanation for this phenomenon.

It was noted above that the metal ion specificity in the Me–citrate complexes transported by two *B. subtilis* transporters, *Bs*CitM and *Bs*CitH, correlated with the ionic radius of the metal ions. *Bs*CitM transporting Mg^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+} and Mn^{2+} with atomic radii ranging in size between 65 and 80 pm would accept the smaller ions, whereas *Bs*CitH transporting Ca^{2+}, Sr^{2+}, and Ba^{2+} with radii ranging from 99 to 134 pm would accept the larger ions [7]. As, in addition, the specificity of the transporters did not correlate with the complexes being bidentate or tridentate [7,19], the size criterion suggests a subtle interaction with the substrates based on the physical size of the binding pocket. The newly identified metal ions Cd^{2+} and Pb^{2+} (radii of 97 and 119 pm, respectively) that are transported by *Bs*CitH as well as *Ef*CitH are in line with the hypothesis. Also, the lack of activity of the two transporters with Fe^{2+}–citrate (radius 76 nm) and Fe^{3+}–citrate supports the hypothesis. The present study of the ion specificity of *Bs*CitH of *B. subtilis* in RSO membranes revealed two differences relative to the previous study employing whole cells that suggest a shift in the range of ionic radii that are accepted by the Ca^{2+}–citrate transporter. At the upper limit, Ba^{2+} (134 pm) is no longer accepted, whereas, at the lower limit, Mn^{2+} (80 pm) is accepted. This subtle shift in the size window may be a reflection of the somewhat

different physicochemical environment of the transporter in the cellular membrane compared with the membrane of an RSO vesicle. Such small changes in the interaction between the substrate and the transporter are also suggested by the observed difference in affinity of the *Bs*CitH transporter for the Ca^{2+}–citrate complex in the two experimental systems. The K_m values in cells and RSO membranes were found to be 33 μM [7] and 1.7 μM (unpublished results), respectively. The ionic radii of Mn^{2+} (80 pm) and Cu^{2+} (73 pm) are both at the lower limit of the size window, which may explain the different activities of *Ef*CitH and *Bs*CitH with these ions (Fig. 6). Small differences in the amino-acid side chains that form the binding pocket may be responsible. The activity of *Bs*CitH with the Cu^{2+}–citrate complex shows that, by itself, Cu^{2+} does not inhibit PMF generation nor has it any other deleterious effect on the membrane. Therefore, the lack of transport of citrate by the membranes containing *Ef*CitH and of proline by all membranes in the presence of Cu^{2+} must be at the level of the transporters themselves. The lack of transport activity of the proline transporter in the presence of Cu^{2+} is most likely due to oxidation of the transporter [20]. Possibly, the two adjacent cysteine residues at positions 137 and 138 in the primary structure of *Ef*CitH can be oxidized to a disulfide, thereby inactivating the transporter, which gives an alternative explanation for the different specificities of the *E. faecalis* and *B. subtilis* transporters.

Experimental procedures

Bacterial strains, growth conditions, and cloning of *Ef*citH

Escherichia coli strains DH5α and BL21(DE3) were routinely grown in Luria–Bertani broth medium at 37 °C under continuous shaking at 150 r.p.m. When appropriate, the antibiotics kanamycin and carbenicillin were added at a final concentration of 50 $\mu g \cdot mL^{-1}$.

All genetic manipulations were performed in *E. coli* DH5α. *Ef*citH was produced in *E. coli* BL21(DE3) harboring plasmid pET-*Ef*citH (see below), which contains the gene coding for *Ef*CitH with an N-terminal His-tag. The cells were induced for 45 min by adding 0.25 mM isopropyl β-D-thiogalactopyranoside when the D_{660} of the culture was 0.8. Expression of *Bs*CitH was performed essentially as described previously [7]. *E. coli* BL21(DE3) harboring plasmid pWSKcitH was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside when the D_{660} of the culture was 0.6, after which the cells were allowed to grow for an additional 1 h.

The gene encoding *EfcitH* was amplified by PCR using genomic DNA of *E. faecalis* ATCC 29212 as the template, following a standard protocol. The forward primer introduced an *Nde*I site around the initiation codon of the *EfcitH* gene, and the backward primer introduced an *Eco*RI site downstream of the stop codon. The PCR product was digested with the two restriction enzymes and ligated into the corresponding restriction sites of vector pET28b (Novagen, La Jolla, CA, USA). The resulting plasmid, named pET-*Ef*citH, codes for *Ef*CitH extended with a His-tag at the N-terminus. The sequence of the insert was confirmed (University of Maine, DNA sequencing Facility, EEUU), and the plasmid was subsequently introduced into *E. coli* BL21(DE3).

Preparation of the RSO membrane vesicles

RSO membrane vesicles were prepared by the osmotic lysis procedure as described previously [21]. Membrane vesicles were resuspended in 50 mM Pipes buffer, pH 6.1, rapidly frozen in liquid nitrogen, and then stored at −80 °C. Membrane protein concentration was determined using the *DC* Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA).

SDS/PAGE and immunoblotting

Membrane proteins were separated by SDS/PAGE (12% gel) and transferred on to a poly(vinylidene difluoride) membrane (Roche, Almere, the Netherlands) by semidry electroblotting. His-tagged proteins were detected with a primary anti-His IgG (Amersham BioSciences, Piscataway, NJ, USA) and a secondary anti-mouse antibody coupled to alkaline phosphatase (Sigma, Zwijndrecht, the Netherlands), followed by chemiluminescent detection with CDP-Star (Roche).

Transport assays in whole cells

After transformation, recombinant clones were assayed for expression of *Ef*CitH by measuring citrate uptake in whole cells. Uptake was measured using the rapid filtration method. Cells were diluted to an D_{660} of 1 in 50 mM Pipes, pH 6.1, in a total volume of 100 μL, and equilibrated at 30 °C. [1,5-^{14}C]Citrate (114 $mCi \cdot mmol^{-1}$; Amersham BioSciences) was added at a final concentration of 4.4 μM. Uptake was stopped by the addition of 2 mL ice-cold 0.1 M LiCl, followed by immediate filtration over cellulose nitrate filters (0.45 μm, pore size). The filters were washed once with 2 mL of the 0.1 M LiCl solution and assayed for radioactivity. The background was estimated by adding the radiolabeled substrate to the cell suspension after the addition of 2 mL ice-cold LiCl, immediately followed by filtering and washing.

Transport assays in RSO membranes

PMF-driven uptake

Uptake was measured by the rapid filtration method as described above. RSO membranes vesicles were energized using the potassium ascorbate/phenazine methosulfate electron donor system [22]. Membranes were diluted to a final concentration of 0.2 mg membrane protein·mL^{-1} into 50 mM Pipes, pH 6.1, and incubated at 30 °C. When indicated, EDTA or bivalent metal ions were present in the assay mixture at the indicated concentrations. Under a constant flow of water-saturated air, and with magnetic stirring, 10 mM potassium ascorbate and 100 μM phenazine methosulfate (final concentrations) were added, and the PMF was allowed to develop for 2 min. Then [1,5-^{14}C]citrate (114 mCi·$mmol^{-1}$) or L-[4-^{14}C]proline (260 mCi·$mmol^{-1}$; Amersham Pharmacia) was added at final concentrations of 4.4 μM and 1.72 μM, respectively.

Affinity measurements

The kinetic constants were derived from initial rates of PMF-driven uptake determined during the first 10 s. The assays were performed in triplicate. The assay buffer contained 1 mM EDTA, 1.5 mM Ca^{2+} and a series of [1,5-^{14}C]citrate concentrations of 0.55, 1.1, 2.2, 4.4 and 8.8 μM. The corresponding concentrations of the Ca^{2+}–citrate complex in the buffer were 87% of the total citrate concentrations. Speciation of the bivalent cations in the transport buffer was calculated using the MINTEQA2 program [23]. K_m and V_{max} values were obtained from a double-reciprocal plot of the rate versus complex concentration.

Homologous exchange and efflux

RSO membrane vesicles were allowed to accumulate radiolabeled [1,5-^{14}C]citrate driven by the electron donor system potassium ascorbate/phenazine methosulfate for 5 min as described above. The PMF was dissipated by the addition of the uncoupler CCCP at a concentration of 10 μM. When indicated, at the same time, 500 μM unlabeled citrate or 1 mM EDTA was added. The release of label from the membranes was followed for 4 min by rapid filtration at various time points.

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